## Materials and Methods

### Materials

Reagents were obtained from the following sources: antibody to raptor from Upstate/Millipore; HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, RagA, RagB, RagC and RagD, mTOR, rictor, phospho-T398 dS6K, and the myc epitope from Cell Signaling Technology; an antibody to the HA tag from Bethyl laboratories; an antibody to RagB from Novus Biologicals; Cellulose PEI TLC plates, RPMI, FLAG M2 affinity gel, FLAG M2 antibody, ATP, cycloheximide, valinomycin, $H_20_2$, 2-deoxyglucose, amino acids, and human recombinant insulin from Sigma Aldrich; DSP, protein G-sepharose, and immobilized glutathione beads from Pierce; DMEM from SAFC Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; Alexa 488 or Texas-Red-X conjugated secondary antibodies, Schneider's media, Express Five Drosophila-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen; and amino acid-free RPMI, amino acid- and phosphate- free RPMI, and amino acid-free Schneider's media from US Biological. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

### Cell lines and tissue culture

HEK-293E, HEK-293T, and HeLa cells were cultured in DMEM with 10% IFS. In HEK-293E, but not HEK-293T, cells the mTORC1 pathway is strongly regulated by serum and insulin (*1*). The HEK-293E cell line was kindly provided by John Blenis (Harvard Medical School).

### Leucine or amino acid starvation and stimulation of cells

Almost confluent cultures in 10 cm culture plates were rinsed with leucine-free RPMI once, incubated in leucine-free RPMI for 50 minutes, and stimulated with 52 µg/ml leucine for 10 minutes. For amino acid starvation, cells in 10 cm culture dishes or coated glass cover slips were rinsed with and incubated in amino acid-free RPMI for 50 minutes, and stimulated with a 10X amino acid mixture for 3-30 minutes as indicated in the figures. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. Cells were processed for biochemical or immunofluorescence assays as described below. The 10X amino acid mixture was prepared from individual amino acid powders.

### Cell lysis, immunoprecipitations, and kinase assays

Cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, and 0.3% CHAPS or 1% Triton X-100, and one tablet of EDTA-free protease inhibitors (Roche) per 25 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 minutes by centrifugation in a microfuge. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 hours at 4°C. 60 µl of a 50% slurry of protein G-sepharose was then added and the incubation continued for an additional 1 hour. Immunoprecipitates were washed three times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 20 µl of sample buffer and boiling for 5 minutes, resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting as described (*2*). For FLAG and GST purifications, immobilized glutathione or Flag M2 affinity resins were washed with lysis buffer 3 times. 20 µl of a 50% slurry of the resins was then added to pre-cleared cell lysates and incubated with rotation for 2 hours at 4°C. Finally, the beads were washed 3 times with lysis buffer containing 150 mM NaCl. For elution of FLAG-tagged proteins, beads were incubated in elution buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 0.5% CHAPS, 50

μg/μl FLAG peptide) for 30 min at 30 °C. Elution of proteins from glutathione beads and kinase assays were performed as previously described (*1*).

**cDNA manipulations and mutagenesis**

The cDNAs for human RagA, RagB, RagC and RagD were obtained from Open Biosystems. The cDNAs were amplified by PCR and the products were subcloned into Sal I and Not I sites of HA-GST-pRK5, HA-pRK5 and FLAG-pRK5. The cDNAs were mutagenized using the QuickChange XLII mutagenesis kit (Stratagene) with oligonucleotides obtained from Integrated DNA Technologies. All constructs were verified by DNA sequencing. The Rag mutants used in our experiments are: $RagB^{GTP}$ = Q99L RagB; $RagB^{GDP}$ = T54L RagB; $RagC^{GTP}$ = Q120L RagC; $RagC^{GDP}$ = S75L RagC; $RagD^{GTP}$ = Q121L RagD; and $RagD^{GDP}$ = S77L RagD. $Rheb1^{GTP}$ = Q64L Rheb1.

FLAG-tagged wild type and mutant RagB and RagD cDNAs or GFP-tagged Rheb1 were amplified by PCR and cloned into the Age I and Bam HI sites of a modified pLKO.1 vector having a CMV promoter (pLJM1). After sequence verification, these plasmids were used, as described below, in transient cDNA transfections or to produce the lentiviruses needed to generate cell lines stably expressing the proteins. An expression plasmid encoding DsRed-Rab7 was obtained from Addgene.

**cDNA transfection-based experiments**

For co-transfection experiments, 2 million HEK-293T or HEK-293E cells were plated in 10 cm culture dishes. 24 hours later, cells were transfected with the prk5-based cDNA expression plasmids indicated in the figures in the following amounts: 500 ng myc-mTOR (*3*); 50 ng myc- or HA-raptor (*2*); 100 ng myc-rictor (*4*); 100 ng HA-GST-, HA- or FLAG-tagged Rap2A (*1*); 100 ng HA-GST-, HA-, or FLAG-tagged Rheb1 (*1*); 100 ng HA-tagged RagA; 100 ng HA-GST- or HA-tagged RagB; 100 ng HA-GST- or HA-tagged RagC; 100 ng HA-GST- or FLAG-tagged RagD; 1 ng of FLAG-S6K1. Transfection mixes were taken up to a total of 2 μg of DNA using empty pRK5.

**In-cell cross-linking assay**

DSP was dissolved in DMSO to a final concentration of 250 mg/ml to make a 250X stock solution for the in-cell cross-linking assay. HEK-293T cells stably expressing FLAG-tagged RagB, $RagB^{GTP}$, or RagD and growing in 10 cm culture dishes were starved for amino acids or starved and then stimulated as described above. At the end of the starvation or stimulation period, cells were rinsed once with cold PBS and incubated with 4 ml of PBS containing 1 mg/ml DSP for 7 minutes at room temperature. The cross-linking reaction was quenched by adding 1M Tris pH 8.5 to a final concentration of 100 mM followed by a 1-minute incubation. The cells were rinsed once with ice cold PBS, and lysed with RIPA buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 1% sodium deoxycholate, 1% NP40, 0.1% SDS, and one tablet of EDTA-free protease inhibitors per 25 ml). FLAG-immunoprecipitates were prepared as described above.

**Identification of RagC as a raptor-associated protein**

FLAG-raptor was immunoprecipitated with the FLAG M2 affinity gel from 30 million HEK-293T cells stably expressing FLAG-raptor. Proteins eluted with the FLAG peptide from the affinity matrix were resolved by SDS-PAGE, stained with silver, and the 45-55 kD region of the gel excised and digested with trypsin overnight. The resulting peptides were separated by liquid chromatography (NanoAcquity UPLC, Waters) using a self-packed Jupiter 3 micron C18 column. The eluting peptides were mass analyzed prior to collisionally induced dissociation (CID) using a ThermoFisher LTQ linear ion trap mass spectrometer equipped with a nanospray

source. Selected mass values from the MS/MS spectra were used to search the human segment of the NCBI non-redundant protein database using Xcalibur Mass Spectrometry software (Thermo Fischer Scientific). In 4 independent purifications of FLAG-raptor performed as above, this procedure led to the identification of a total of 20 peptides matching RagC that were not present in control FLAG-tubulin samples.

**Cell size determinations**

To measure cell size, 2 million cells were plated into 10 cm culture dishes. 24 hours later the cells were harvested by trypsinization in a 4 ml volume, diluted 1:20 with counting solution (Isoton II Diluent, Beckman Coulter), and cell diameters determined using a particle size counter (Coulter Z2, Beckman Coulter) with Coulter Z2 AccuComp software.

**Mammalian lentiviral shRNAs**

TRC lentiviral shRNAs (*5*) targeting RagA, RagB, RagC and RagD were obtained from Sigma. The TRC number for each shRNA is as follows:

Human RagA shRNA #1: TRCN0000047305
Human RagA shRNA #2: TRCN0000047307
Human RagB shRNA #1: TRCN0000047308
Human RagB shRNA #2: TRCN0000047311
Human RagC shRNA #1: TRCN0000072874
Human RagC shRNA #2: TRCN0000072877
Human RagD shRNA #1: TRCN0000059533
Human RagD shRNA #2: TRCN0000059534

The shLuc and shGFP control shRNAs and the shRNAs targeting mTOR and raptor are previously described and validated (*6*). Lentiviral shRNAs targeting Rheb1 were cloned into LKO.1 vector as described (*6*). The target sequences for the Rheb1 shRNAs are:

Human Rheb1 shRNA#1: CCTCAGACATACTCCATAGAT
Human Rheb1 shRNA#2: TTATGTTGGTTGGGAATAAGA

shRNA-encoding plasmids were co-transfected with Delta VPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T cells using FuGENE 6 transfection reagent as previously described (*6, 7*). Virus-containing supernatants were collected 48 hours after transfection, filtered to eliminate cells and target cells were infected in the presence of 8 μg/ml polybrene. 24 hours later, cells were selected with puromycin and analyzed on the 2$^{nd}$ or 3$^{rd}$ day after infection.

**In vitro Rag-raptor binding assay**

2 million HEK-293T cells were plated into 10 cm culture dishes. 24 hours later, the cells were transfected with 2 μg HA-GST-Rap2a, 2 μg HA-GST-Rheb1, or 2 μg HA GST-RagB together with 2 μg of HA GST-RagC (wild type or Rag mutants as indicated). 2 days after transfection, the cells were lysed in Rheb lysis buffer containing 1% Triton X-100 (*1*), and cleared lysates were incubated with glutathione beads for 1.5 hours at 4°C with rotation. The beads were washed 3 times with lysis buffer and two times with Rheb storage buffer (*1*). 1/4 of the glutathione beads were incubated with 300 ng of FLAG-raptor in Rheb lysis buffer with 0.3% CHAPS for 30 min at 4°C with rotation. The glutathione beads were washed twice with Rheb lysis buffer containing 0.3% CHAPS and proteins were denatured by the addition of 20 μl of sample buffer and boiling

for 5 minutes and analyzed by SDS-PAGE and immunoblotting. FLAG-raptor was purified from HEK-293T cells stably expressing FLAG-raptor. Following the affinity-tag purification, the protein was further purified as described (*1*) by gel filtration using a Superose 6 10/300GL column (GE Healthcare) to obtain FLAG-raptor that by silver and coomasie staining was 99% pure.

**Determination of guanyl nucleotide binding state of RagB in cells**

Determination of the guanyl nucleotides bound to RagB in cells was performed essentially as previously described for Rheb (*8*)**.** HEK-293T cells were cultured in fibronectin coated 6-well dishes until confluent, rinsed once with phosphate-and serum-free DMEM, and then incubated in phosphate-and serum-free DMEM containing 1 mCi $^{32}P$ orthophosphate (Perkin Elmer) for 4 hours. After 4 hours, cells were rinsed once with serum-, amino acid-, and phosphate-free RPMI, incubated with serum-, amino acid-, and phosphate-free media containing 1 mCi $^{32}P$ orthophosphate for 50 minutes, and stimulated with amino acids as described above. After rinsing with ice-cold PBS, cells were lysed in 0.5 ml lysis buffer (50 mM HEPES KOH (pH 7.4), 100 mM NaCl, 1 mM $KH_2PO_4$, 1 mM ATP, 100 µM GDP, 100 µM GTP, 5 mM $MgCl_2$, 1% Triton X-100), and the lysate microcentrifuged for 10 minutes at 13,000 rpm at 4°C. The supernatant was incubated with 15 µl of RagB antibody (Novus Biologicals) for 1 hour at 4°C. 20 µl of protein G beads were added to the lysates and the incubation continued for another 45 minutes. The beads were washed 8 times with wash buffer (50 mM HEPES KOH [pH 7.4], 500 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 0.005% SDS) and bound nucleotides were eluted in 23 µl of elution buffer (10 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GDP, 0.5 mM GTP) at 60 °C for 10 minutes. 15 µl of the eluate was transferred to a clean tube. 60 µl of methanol and then 30 µl of chloroform were added to the tube, each addition followed by brief vortexing and microcentrifugation. After the addition of 45 µl of water, the samples were vortexed vigorously, microcentrifuged for 1 minute at 13,000 rpm and the aqueous phase transferred to a clean tube. Using a speedvac the samples were dried and then re-suspended in 15 µl of deionized water. Samples were spotted on PEI cellulose TLC plates and the plates developed with 1.2 mM ammonium formate and 0.8 mM HCl, and the radioactivity detected with a phosphoimager. After background subtraction, the intensities of the GTP and GDP spots were determined. The percent of GTP nucleotide was calculated using the following formula: [(GTP/3)/((GTP/3) + (GDP/2))] X 100%.

**Immunofluorescence assays**

150,000 HEK-293T cells were plated on fibronectin-coated glass coverslips in 12-well tissue culture plates. 24 hours later cells were starved for and stimulated with amino acids as described above, rinsed with PBS once and fixed for 5 minutes with 4% paraformaldehyde in PBS warmed to 37°C. The coverslips were rinsed twice with PBS and permealized with 0.2% Triton X-100 in PBS for 15 minutes. After rinsing twice with PBS, the coverslips were blocked for one hour in blocking buffer (0.25% BSA in PBS) and incubated with primary antibody in blocking buffer overnight at 4°C, rinsed twice with blocking buffer and incubated with secondary antibodies (diluted in blocking buffer 1:1000) for one hour at room temperature in dark. The coverslips were mounted on glass slides using Vectashield (Vector Laboratories) and imaged with a 63X objective using epifluorescence microscopy.

Where indicated, 1-2 ng of plasmids encoding GFP-Rheb1 or dsRed-Rab7 were transfected per well of a 12-well dish.

## *Drosophila* cell experiment

dsRNA transfection and amino acid starvation and stimulation

5 million S2 cells were plated in 6-cm culture dishes in 5 ml of Express Five SFM media. Cells were transfected with 1 µg of dsRNA per million cells. 4 days later, cells were rinsed once with amino acid-free Schneider's medium, and starved for amino acids by replacing the media with amino acid-free Schneider's medium for 1.5 hours. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider's medium for 20 minutes. Cells were then washed with ice cold PBS twice, lysed, and processed as described (*1*). The pH of the amino acid free media was made to match that (pH 6.70) of an opened bottle of full Schneider's media.

Design and synthesis of dsRNAs

The control GFP dsRNA has been described (*4*). Other dsRNAs were designed to target all known transcripts of the target *Drosophila* gene. In order to minimize off-target effects, we used the DRSC tool at http://flyrnai.org/RNAi_find_frag_free.html and excluded regions of 19-mer-or-greater identity to any *Drosophila* transcripts. The Drosophila genome encodes for one RagA/B-like gene (CG11968) and one RagC/D-like gene (CG8707). We designed and tested two distinct dsRNAs to each gene. Synthesis of dsRNAs was performed as previously described (*4*) using the primers indicated below.

Primer sequences (including underlined 5' and 3' T7 promoter sequences)

dRagB (CG11968) dsRNA_1 forward primer:
GAATTAATACGACTCACTATAGGGAGACGCTCCATTATCTTTGCTAACTATAT

dRagB (CG11968) dsRNA_1 reverse primer:
GAATTAATACGACTCACTATAGGGAGACATCAAACACATAAATCAGCACTTC

dRagB (CG11968) dsRNA_2 forward primer:
GAATTAATACGACTCACTATAGGGAGAATAGAGCGCGACATCCATTACTAC

dRagB (CG11968) dsRNA_2 reverse primer:
GAATTAATACGACTCACTATAGGGAGACTTGATGATGTTGGACACCTTTT

dRagC (CG8707) dsRNA_1 forward primer:
GAATTAATACGACTCACTATAGGGAGAGAGTCGACCAGTAAGATCGTGAA

dRagC (CG8707) dsRNA_1 reverse primer:
GAATTAATACGACTCACTATAGGGAGATGTAGTCATCCTTGGCATCG

dRagC (CG8707) dsRNA_2 forward primer:
GAATTAATACGACTCACTATAGGGAGAAGTTCGAGGTGTTTATACACAAGGT

dRagC (CG8707) dsRNA_2 reverse primer:
GAATTAATACGACTCACTATAGGGAGAAAGATGGAGTGGTCGTATATGGAG

**References for Materials and Methods**


1. Y. Sancak et al., *Mol Cell* 25, 903-15 (2007).
2. D.-H. Kim et al., *Cell* 110, 163-175 (2002).
3. P. E. Burnett, R. K. Barrow, N. A. Cohen, S. H. Snyder, D. M. Sabatini, *PNAS* 95, 1432-1437 (1998).
4. D. D. Sarbassov et al., *Curr Biol* 14, 1296-302 (2004).
5. J. Moffat et al., *Cell* 124, 1283-98 (2006).
6. D. D. Sarbassov, D. A. Guertin, S. M. Ali, D. M. Sabatini, *Science* 307, 1098-101 (2005).
7. S. M. Ali, D. M. Sabatini, *J Biol Chem* 280, 19445-8 (2005).
8. X. Long, Y. Lin, S. Ortiz-Vega, K. Yonezawa, J. Avruch, *Curr Biol* 15, 702-13 (2005).

# Figure S1

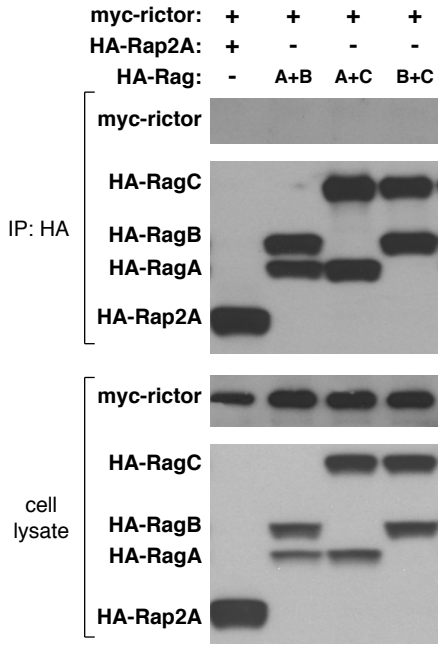


**Fig. S1.** Rag heterodimers do not interact with rictor. HEK-293T cells were transfected with the indicated cDNAs in expression vectors, cell lysates prepared, and lysates and HA-immunoprecipitates analyzed for the levels of the specified proteins. This experiment was performed side-by-side with that shown in Figure 1A and is displayed here for space reasons.

# Figure S2

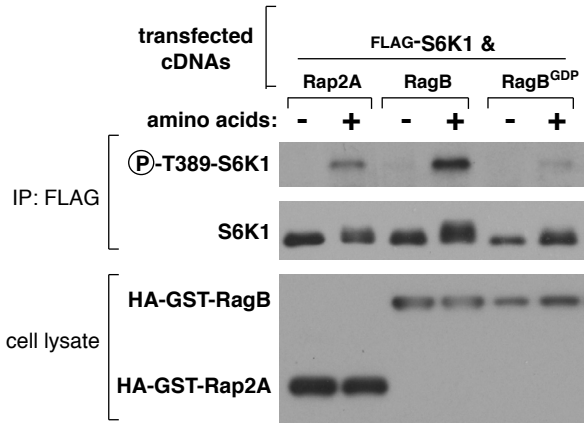


**Fig S2.** Expression of RagB$^{GDP}$ alone suppresses endogenous mTORC1 signaling. Cell lysates were prepared from HEK-293T cells transfected with the indicated cDNAs and deprived for 50 minutes of serum and amino acids, and where indicated, stimulated with amino acids for 10 minutes. Lysates and FLAG-immunoprecipitates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1.

# Figure S3

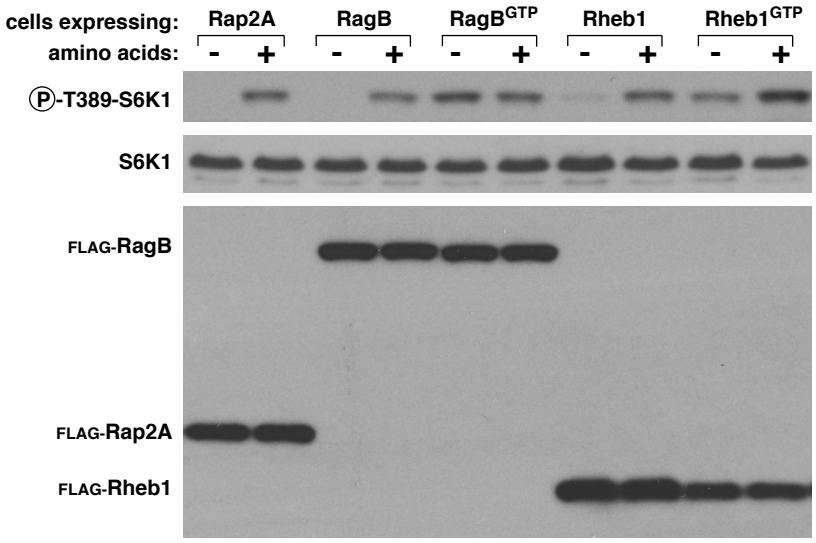


**Fig S3.** Stable expression of Rheb1 or Rheb1$^{GTP}$ does not eliminate the sensitivity of the mTORC1 to amino acids. HEK-293T cells stably expressing the indicated proteins were deprived of serum and amino acids for 50 minutes, and where indicated, stimulated with amino acids for 10 minutes. Lysates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1.

# Figure S4

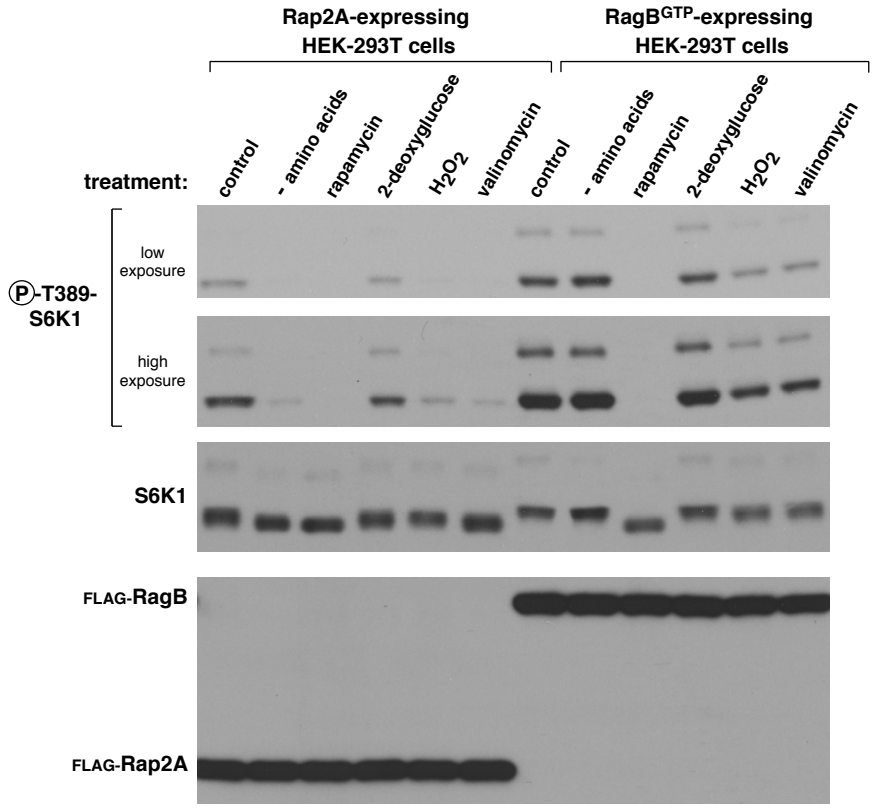


**Fig. S4.** Effects of various insults on mTORC1 signaling in cells expressing $RagB^{GTP}$. HEK-293T cells stably expressing Rap2A or $RagB^{GTP}$ and growing in full media were untreated (control), or deprived of amino acids for 30 minutes, or treated for 30 minutes with 20 nM rapamycin, 100 mM 2-deoxyglucose (energy deprivation), 1 mM $H_2O_2$ (oxidative stress), or 1 μM valinomycin (mitochondrial proton gradient inhibition). Cell lysates were prepared and analyzed for the levels of the specified proteins and the phosphorylation state of S6K1.

# Figure S5

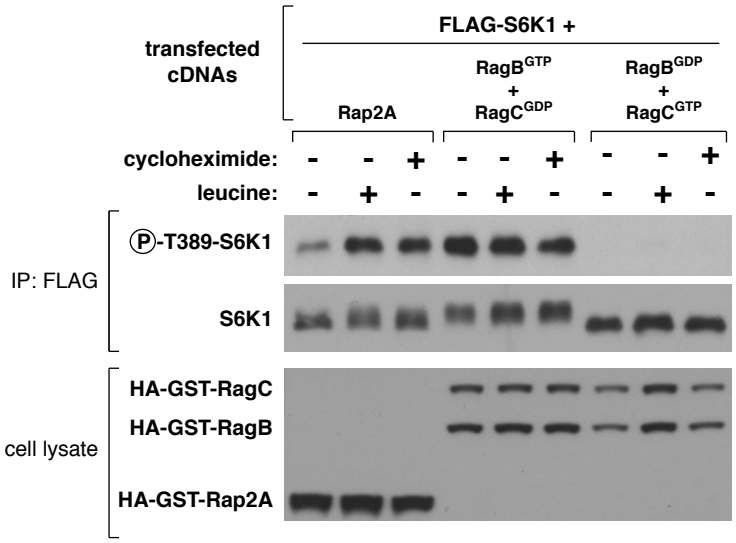


**Fig. S5.** Inhibition of protein synthesis with cycloheximide does not prevent $RagB^{GDP}$-$C^{GTP}$ from inhibiting the mTORC1 pathway. HEK-293T cells were transfected with the indicated cDNAs in expression vectors, and starved for 50 minutes for serum and leucine, and, where indicated, stimulated with leucine or 10 μg/ml cycloheximide for 20 minutes. Cell lysates and FLAG-immunoprecipitates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1.

# Figure S6

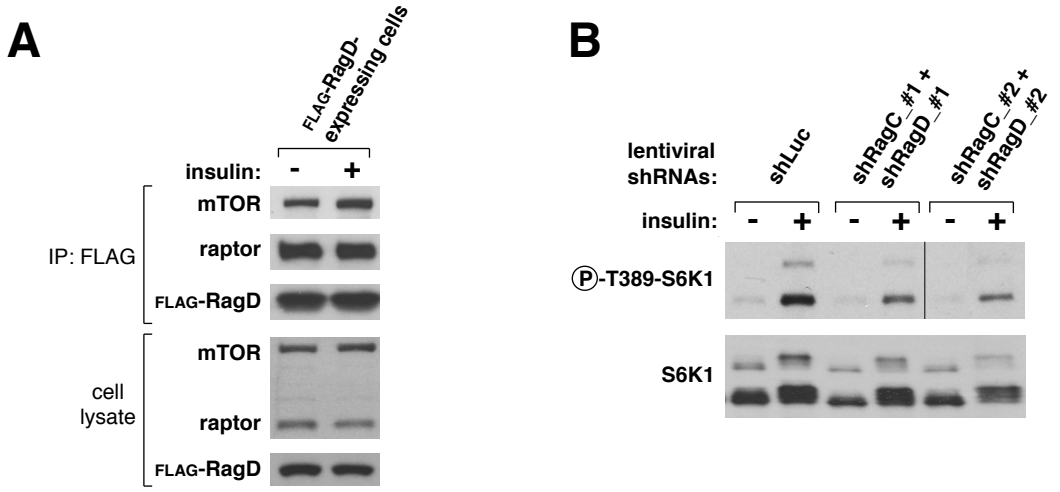


**Fig. S6. (A)** Insulin does not increase the interaction between mTORC1 and the Rag proteins. Hela cells stably expressing FLAG-RagD were starved for serum for 50 minutes and, where indicated, stimulated with 100 nM insulin for 10 minutes. Cells were then processed with a chemical cross-linking assay and cell lysates and FLAG-immunoprecipitates analyzed for the levels of the indicated proteins. **(B)** Knockdowns of RagC and RagD suppress insulin-stimulated phosphorylation of S6K1. HeLa cells expressing shRNAs targeting RagC and RagD were prepared as in Figure 3. Cells were deprived of serum for 50 minutes, and, where indicated, re-stimulated with 100 nM insulin for 10 minutes. Cell lysates were prepared and analyzed for the levels and phosphorylation state of S6K1.

# Figure S7

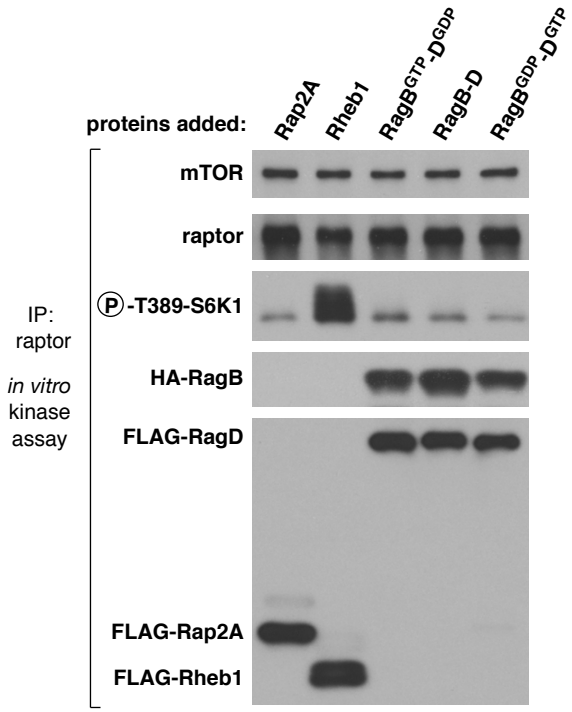


**Fig. S7.** Rag heterodimers do not activate the in vitro kinase activity of mTORC1 in assays in which Rheb1 does. In vitro kinase assays containing 300 ng of each of the indicated proteins or Rag heterodimers were performed as described in the methods using mTORC1 immunopurified from HEK-293T cells with a raptor antibody. Rap2A, Rheb1, and the Rag heterodimers were expressed in HEK-293T cells and purified under conditions where the endogenous nucleotides remained bound.

# Figure S8

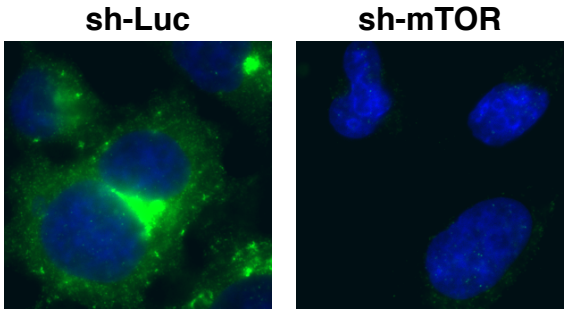


**Fig. S8.** Validation that an mTOR antibody detects mTOR in an immunofluorescence assay. HEK-293T cells expressing a control shRNA (sh-Luc) or an shRNA targeting mTOR (sh-mTOR) that is known to reduce mTOR expression (see methods) were processed in an immunofluorescence assay with an mTOR antibody (green) and co-stained with DAPI for DNA content (blue).

# Figure S9

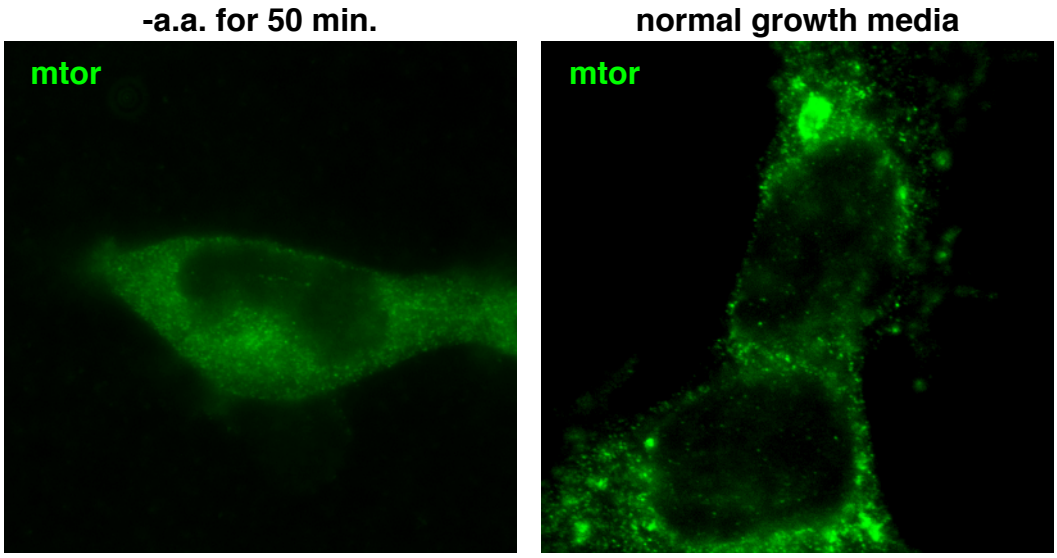


**Fig. S9.** Localization of mTOR in HEK-293T cells growing in normal growth media. Where indicated, cells were deprived of amino acids for 50 minutes. For cells growing in normal media, the media was replaced with fresh media 1 hour before processing. Cells were processed in an immunofluorescence assay with an mTOR antibody (green) and imaged.

# Figure S10

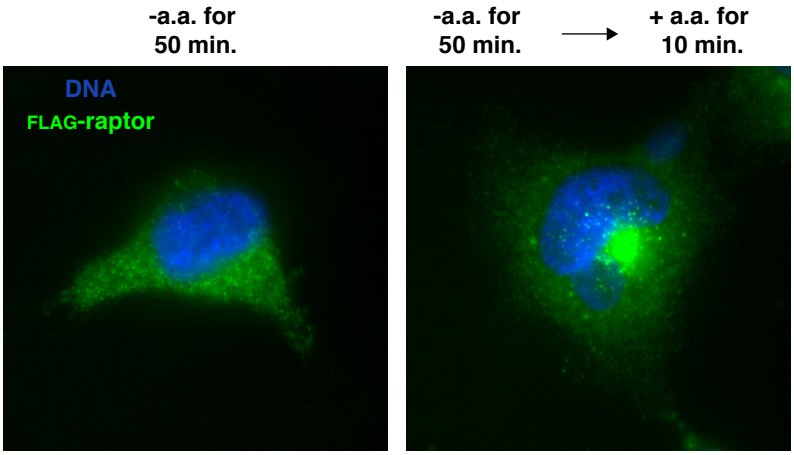


**Fig. S10.** Raptor localization in cells deprived or stimulated with amino acids. HEK-293T cells stably expressing FLAG-raptor were deprived of serum and amino acids for 50 minutes, and where indicated, stimulated with amino acids for 10 minutes. Cells were processed in an immunofluorescence assay with a FLAG antibody (green), co-stained with DAPI for DNA content (blue), and imaged.

## Figure S11

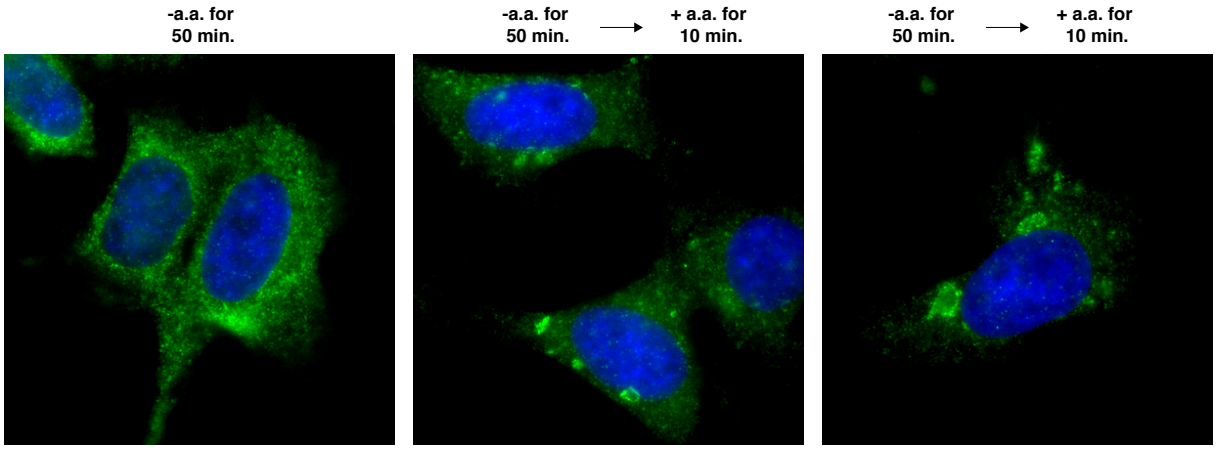


**Fig. S11.** mTOR localization in HeLa cells starved and stimulated for amino acids as in Figure 4. Cells were processed in an immunofluorescence assay with an mTOR antibody (green) and co-stained with DAPI for DNA content (blue).

# Figure S12

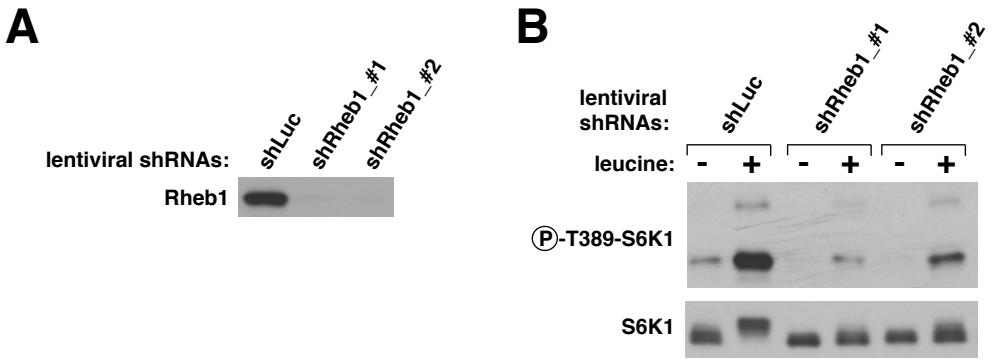


**Fig. S12.** Rheb is necessary for leucine to activate the mTORC1 pathway. **(A)** Validation that the two independent shRNAs targeting Rheb1 reduce its expression. Immunoblot analysis of Rheb1 in HEK-293T cells expressing the indicated shRNAs. **(B)** Cells from (A) were deprived of serum and leucine for 50 minutes, and, where indicated, re-stimulated with leucine for 10 minutes. Cell lysates were prepared and analyzed for the levels and phosphorylation state of S6K1.